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Evaluation of LMH cell line to study chicken Toll-like receptor functions

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Für alle Schafe und Esel dieser Welt

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List of Abbreviations

bp	base pairs
BoHV-1	Bovine Herpesvirus-1
chCAF	chicken Chemotactic and Angiogenic Factor
chCXCLi	chicken CXC Ligand
CXCR	CXC Receptor
DEPC	Diethylpyrocarbonate
FACS	Fluorescence Activated Cell Sorting
GaHV-1	Gallid Herpesvirus-1
GAPDH	Glutaradehyde3-Phosphate Dehydrogenase
HSV-1	Herpes Simplex Virus-1
IFN	Interferon
IL	Interleukin
ILTV	Infectious Laryngotracheitis Virus
i.m.	Intramuscular
LMH	Leghorn Male Hepatoma cell line
LPS	Lipopolysaccharid (endotoxin)
LRR	Leucine-Rich Repeats
NFκB	Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
p.i.	post infectionem
Poly I:C HMW	Polyinosinic:polycytidylic acid High Molecular Weight
Poly I:C LMW	Polyinosinic:polycytidylic acid Low Molecular Weight
ppm	parts per million
PRR	Pattern Recognition Receptor
(m/ss/ds)RNA	(messenger/single stranded/double stranded) Ribonucleic Acid
TIR	Toll/IL-1 Receptor domain
TLR	Toll-like Receptor

1. Abstract

1.1 Abstract

The aim of this study was to analyse Toll-like receptor (TLR) function in Leghorn male hepatoma (LMH) cells infected with Gallid Herpesvirus-1 (GaHV-1). To date, the chicken innate immune system is poorly understood and nothing is known about GaHV-1 and its interaction with TLRs.

GaHV-1 belongs to the Alphaherpesvirinae and causes the economically important fowl disease infectious laryngotracheitis. Symptoms vary from loss in egg production to severe respiratory lesions and acute death.

In order to analyse the potential of LMH cells as a tool for studying TLR functions the presence of TLR 3, 7 and 21 first had to be demonstrated. Thus, five novel real-time PCR based assays detecting TLR 3, 7 and 21 and additionally the interleukins 6 and 8 were established. After proving the presence of these mRNAs in LMH cells, the influence of infection with GaHV-1 on the mRNA expression was examined. Infection with GaHV-1 reduced the mRNA expression of TLR 3 and 7. No conclusive results were obtained concerning TLR 21. Interestingly, IL-6 mRNA expression was only detectable in cells being infected more than 24 hours. In contrast, IL-8 mRNA was expressed in all samples and also increased 24 hours p.i. Further, LMH cells were treated with TLR agonists, but no conclusive results were obtained other than all cells treated with TLR 3 agonists died.

In summary, this study provides the first experiments analysing the influence of GaHV-1 infection on TLR and cytokine mRNA expression in LMH cells.

Keywords: LMH cells / Gallid Herpesvirus-1 / Toll-like receptor

1.2 Zusammenfassung

Das Ziel dieser Studie war die Analyse der Toll-like Rezeptorfunktionen in Leghorn male hepatoma (LMH) Zellen. Dafür wurden sie mit Gallid Herpesvirus-1 (GaHV-1) infiziert. GaHV-1 gehört zu den Alphaherpesvirinae und verursacht die ökonomisch wichtige Geflügelkrankheit infektiöse Laryngotracheitis. Die Symptome variieren von verminderter Legeleistung, schweren Läsionen im Atemtrakt bis zu akutem Tod. Bis heute ist das innate Immunsystem des Huhnes wenig erforscht und es ist nichts über die Interaktion zwischen GaHV-1 und den TLRs bekannt.

Um die LMH Zelllinie als geeignetes Werkzeug für TLR Studien zu validieren, musste zuerst die Existenz von TLR 3, 7 und 21 nachgewiesen werden. Dafür wurden, zusätzlich zu den TLRs auch für IL-6 und IL-8 neue real-time PCR Assays etabliert. Nachfolgend wurde der Einfluss der GaHV-1 Infektion auf die mRNA Expression ermittelt. Die Infektion mit GaHV-1 reduzierte die mRNA Expression von TLR 3 und 7. Die Messung der TLR 21 mRNA lieferte keine schlüssigen Resultate. IL-6 mRNA war nur in Zellen detektierbar, die länger als 24 Stunden infiziert waren. IL-8 mRNA war in allen Proben nachweisbar. In weiteren Experimenten wurden die Zellen mit TLR Agonisten stimuliert. Dabei konnten keine schlüssigen Resultate gewonnen werden, ausser, dass alle Zellen, die mit einem TLR 3 Agonisten stimuliert wurden, starben.

Diese Studie dokumentiert die ersten Experimente, die den Einfluss von GaHV-1 auf die Expression von TLR- und Zytokin- mRNA in LMH Zellen messen.

Stichworte: LMH Zellen / Gallid Herpesvirus-1 / Toll-like Rezeptor

2. Introduction

2.1 The Leghorn Male Hepatoma cell line (LMH)

The continuous cell line LMH was established by Kawaguchi and coworkers in 1987. A hepatic tumour was induced in a male leghorn chicken by long-term treatment with diethylnitrosamine. Chickens were given drinking water containing 100ppm diethylnitrosamine for 9 weeks and then i.m. injections of 20 - 100mg diethylnitrosamine/kg for another 30 weeks. At week 24 one animal showing a hepatic tumour was dispatched. Cells were cultured from this tumour.

The LMH cell line was established as the first chicken epithelial cell line. It was new that tumour induction in chicken was done chemically and not using a virus (Kawaguchi et al. 1987). The LMH cell line is useful for research in biological parameters and carcinogenesis in chicken (Kawaguchi et al. 1987). In general, it is suitable for transfection studies (LMH product description, ATCC). The chemically induced tumour cells are described as the only suitable continuous cell line for virus infection and propagation, although the need for adaptation of the virus is known (Schnitzlein et al. 1994, Scholz et al. 1993)

2.2 Toll-like receptors (TLR)

2.2.1 Toll-like receptors in general

The innate immune system represents the first line of defence against various pathogens (Juul-Madsen et al. 2008, Martinez-Martin and Viejo-Borbolla 2010). Early recognition occurs through a number of germ-line encoded pattern-recognition receptors (PRRs) such as scavenger receptors, Toll-like receptors etc. (Brownlie and Allan 2011, Juul-Madsen et al. 2008). Ligands for these receptors are named pathogen associated molecular patterns (PAMPs). These PAMPs are invariant motifs of the pathogen, which are essential for survival of the pathogen but different from the host's motifs (Takeda and Akira 2005). Toll-like receptors form a highly conserved group of PRRs. Their role in host defence was described the first time by Lemaitre and coworkers in 1996. Actually the Toll gene and its protein product were recognized as essential factor in embryogenesis of Drosophila melanogaster (Anderson et al. 1985). A description of the Toll protein structure was first given by Hashimoto and coworkers in 1988. The similarity between Drosophila Toll and the

human IL-1 receptor and thereby the existence of these receptors in different species opened a wide field for research (Lemaitre et al. 1996). Toll-like receptors consist of an extracellular N-terminal domain, which contains leucine-rich repeats (LRRs), one or two cysteine rich regions, a transmembrane domain and a highly conserved cytoplasmatic Toll/IL-1 receptor domain (TIR) (Brownlie and Allan 2011, Hashimoto et al. 1988). The N-terminal domain interacts with the PAMP and varies therefore adapted to its use. In contrast, the TIR domain is highly conserved between the TLRs and has a very ancient evolutionary origin (Brownlie and Allan 2011). TLRs can be divided into extracellular receptors, recognizing PAMPs at cell surface and intracellular members, sensing PAMPs that are translocated into the inner compartment of the cell (Brownlie and Allan 2011, Takeda and Akira 2005). Each TLR recognises individual PAMPs from bacteria, protozoa and viruses (Brownlie and Allan 2011, Juul-Madsen et al. 2008).

TLRs play a pivotal role in initiation of the adaptive immune system and secretion of cytokines and chemokines (Juul-Madsen et al. 2008, Martinez-Martin and Viejo-Borbolla 2010). Cytokines and chemokines act as extracellular signals between cells and have various functions in the immune response as for example cell attraction in inflammatory processes (Kaiser et al. 2008). But also negative consequences of TLR mediation are known as for example in the infection of mice with West Nile Virus, where TLR 3 mediates the virus entry into the brain and enables encephalitis (Wang et al. 2004). In mammals, so far 13 Toll-like receptors are known (Uematsu and Akira 2006). At least 11 are expressed in humans, the additional TLRs were found in mice (Finberg et al. 2004).

2.2.2 Chicken Toll-like receptors

Ten avian Toll-like receptors have been discovered so far (Brownlie and Allan 2011). Five of them, TLR 2a and 2b, 3, 4, 5 and 7 are orthologues to mammalian Toll-like receptors (Brownlie and Allan 2011). In contrast, TLR 8 has been genomically disrupted and TLR 9 deleted over time, meaning that they are not expressed in chicken (Brownlie and Allan 2011). Interestingly, chickens have two additional TLRs instead: TLR 15 and TLR 21. TLR 21 seems to be a replacement for TLR 9, since it was shown to recognize unmethylated CpG motifs, the ligand for TLR 9 (Boehme and Compton 2004, Brownlie and Allan 2011). The function and cellular location of avian Toll-like receptors is similar to that in mammals (Brownlie and Allan 2011). For

example TLR 3 senses dsRNA linked with viruses, TLR 7 recognises ssRNA associated with viruses and TLR 4 reacts to LPS of gram-negative bacteria (Boehme and Compton 2004, Brownlie and Allan 2011).

2.2.3 Toll-like receptors and viruses

It was first described in 2000, that a TLR (TLR 4) is involved in defence against viruses (Kurt-Jones 2000). Nowadays, it is known, that different Toll-like receptors are involved in virus recognition, such as TLR 2, 3, 4, 7, 8 and 9 (Boehme and Compton 2004). TLRs 2 and 4, which are located on cell surface, are activated by contact with proteins being expressed on the viral surface (Boehme and Compton 2004, Finberg et al. 2004, Paludan et al. 2011). In contrast, TLR 3, 7, 8 and 9 are placed on endosomes and therefore sense ligands, which are translocated in the intracellular space (Boehme and Compton 2004, Brownlie and Allan 2011, Paludan et al. 2011). Consequence of TLR activation is a signal cascade leading to an orchestrated activation of innate and adaptive immune response (Boehme and Compton 2004). For example TLR 3 activation leads to secretion of proinflammatory cytokines and IFNβ response (Khvalevsky et al. 2007, Takeda and Akira 2004). Proinflammatory cytokines like IL-6 and IL-8 activate the inclusion of lymphocytes into the host defence process (Kaiser et. al. 2008).

2.2.4 Toll-like receptors and human Herpesviruses

In the literature, the interaction between Alpha-, Beta- and Gammaherpesviruses and the Toll-like receptor system is described (Boehme and Compton 2004, Paludan et al. 2011). For example Herpes Simplex virus-1, an Alphaherpesvirus, activates TLR 2, 3 and 9 and human Cytomegaly virus, a Betaherpesvirus, interacts with TLR 2 and 9 (Boehme and Compton 2004, Paludan et al. 2011). The activation of TLRs results in production of antiviral interferons and inflammation modulating cytokines (Takeda and Akira 2004).

2.2.5 Toll-like receptors and animal Herpesviruses

Important representatives of animal Herpesviruses are the equine Herpesviruses 1 and 4 causing abortion and rhinopneumonitis, bovine Herpesvirus 1 causing Infectious Bovine Rhinotracheitis – Infectious Pustular Vulvovaginitis – Infectious

Balanoposthitis (IBR-IPV-IBP) and porcine Herpesvirus 1 causing Aujeszky disease (Rolle and Mayr 2007). Most of the frequently occurring Herpesviruses in animals belong to the Alphaherpesviruses (Davison et al. 2009). Related to Toll-like receptors, the most systematic examined animal model is the mouse using human Herpesviruses (e.g. Carty and Bowie 2010). Research concerning the interaction between the different animal Herpesviruses and their TLRs exist, but it is still at the beginning.

The two known diseases in chickens caused by Herpesviruses are Infectious Laryngotracheitis and Marek's Disease (Rolle and Mayr 2007). Literature concerning the interaction between these viruses and the avian TLR system has hitherto not been reported.

2.3 Interleukins

2.3.1 Interleukins in general

Activation of the TLR receptors initiates the expression of different inflammatory cytokines, interferons and other proteins, which are important for the pathogen control (Brownlie and Allan 2011, Takeda and Akira 2004). Two important and multifunctional mediators in the inflammatory process are IL-6 and IL-8 (Jones 2005, Martins-Green and Feugate 1998, Terasaka et al. 2010). IL-6 is expressed by a various number of cell types and plays a major role in regulating the immune response, acute phase reactions and haematopoiesis (Jones 2005). IL-6 shows remarkably little species specificity (Jones 2005, Schneider et al. 2001). Different studies showed, that IL-6 of one species is able to trigger IL-6 response in other species (Jones 2005, Schneider et al. 2001). Mammal IL-8 (CXCL8) belongs to the chemokines, a family of structurally related cytokines with chemotactic activity for specific types of leukocytes during inflammatory and allergic response. IL-8 is also involved in growth regulation, wound healing and angiogenesis (Kaiser et al. 1999, Martins-Green and Feugate 1998, Sick et al. 2000).

2.3.2 Chicken interleukins

Chickens have a functional homologue of the mammalian IL-6 (Schneider et al. 2001). Concerning IL-8, two CXCL8-like chemokines exist: chCXCLi1 (=K60) and

chCXCLi2 (=9E3/CEF4=chCAF). Both are ligands for the same receptor (CXCR1) but have slightly different functions. ChCXCLi1 is mainly expressed in lymphoid tissue and induces the migration of heterophils. ChCXCLi2 is expressed in lymphoid and non-lymphoid tissue and attracts monocytes and lymphocytes. ChCXCLi2 is the true orthologue of mammalian IL-8 with 51% homology and it is also homologous to some other mammalian chemokines like Groα/MGSA (Kaiser et al. 1999, Martins-Green and Feugate 1998, Poh et al. 2008, Sick et al. 2000).

2.4 Gallid Herpesvirus-1

2.4.1 History and Etiology

The earliest description of Infectious Laryngotracheitis (ILT) in chicken is reported from the United States in March 1920 (Cover 1996). In 1930, the cause of ILT was first described by Beaudette as a filterable virus (Guy and Garcia 2008). Until 1931, ILT was known under different names as for example infectious bronchitis, tracheolaryngitis and avian diphtheria (Cover 1996). In 1931, the Special Committee on Poultry Diseases of the American Veterinary Medical Association adopted the name ILT (Guy and Garcia 2008). In 1963, the causative agent was characterized as a member of the Herpesvirales (Cover 1996). The International Committee on Taxonomy of Viruses has updated the taxonomy of Herpesviruses (Davison et al. 2009). Now, the virus belongs to the order of Herpesvirales, family Herpesviridae, subfamily Alphaherpesvirinae, genus Iltovirus (Davison et al. 2009). Several different strains of the GaHV-1 are described so far (Guy and Garcia 2008, Rolle and Mayr 2007). They differ in virulence but display similar serological properties (Guy and Garcia 2008, Rolle and Mayr 2007).

2.4.2 Structure and replication

GaHV-1 consists of an icosaedral symmetric capsid with a diameter of around 80 - 100nm. The capsid contains 162 hexagonal capsomers and is surrounded by an irregular envelope consisting of a lipid containing double membrane (diameter 195-200nm). The envelope carries glycoprotein spikes, which are important for the interaction between host and virus (Boehme and Compton 2004, Guy and Garcia 2008). Herpesviruses inherit a linear double-stranded DNA as genome with approximately 155’000 base paires (Guy and Garcia 2008). A morphological and

genomic homology between GaHV-1 and various other Alphaherpesviruses is present. The replication cycle of GaHV-1 is comparable to that of other Alphaherpesviruses (Fuchs et al. 2007, Guy and Garcia 2008, Rolle and Mayr 2007). Replication takes place as follows: The viral glycoproteins interfere with cell receptors initiating the fusion of the envelope with the host cell membrane (Guy and Garcia 2008). The nucleocapsid is released into cytoplasm and transported to the nuclear membrane (Guy and Garcia 2008). Subsequently, the viral DNA gets through the nuclear pores into the nucleus (Guy and Garcia 2008). Transcription and replication occurs within the nucleus, using the host's cellular enzymes (Guy and Garcia 2008, Rolle and Mayr 2007). The expression of the viral genes is strictly organized (Ackermann et al. 2007, Guy and Garcia 2008, Rolle and Mayr 2007). Virus encoded proteins with regulatory, activating and suppressive functions are produced in the immediate early phase (Ackermann et al. 2007). After DNA-replication, structural proteins are synthesised and the viral particles become composed and completed in the late phase (Ackermann et al. 2007, Rolle and Mayr 2007). Concerning the release of the viral capsids out of the nucleus, two different theories exist. In 2009 Mettenleiter and coworkers described that the capsids acquire a primary envelope from the inner lamellae of the nuclear membrane. In course of reaching the cell membrane, they gather the tegument in the cytosol and the final envelope with glycoprotein spikes in Golgi-derived vesicles. In contrast, Wild and coworkers (2005, 2009) observed, that BoHV-1 and HSV-1 have the ability to widen the nuclear pores and leave the nucleus passing them. They saw, that the capsids are able to activate the budding process at any cellular membrane. The completed virions are released (elution) by lysis of the cell or by vacuolar membrane fusion and exocytosis (Guy and Garcia 2008).

2.4.3 Pathobiology and epidemiology

Infectious laryngotracheitis (ILT) occurs in most countries all over the world (Bagust et al. 2000, www.bvetadmin.ch). Especially in areas with intensive poultry farming and large concentrations of fowl, the disease displays a big economic importance (Guy and Garcia 2008). In these areas, special biosecurity measures and vaccination are used to control the infection (Guy and Garcia 2008). In Switzerland, ILT represents a notifiable disease and vaccination is therefore forbidden. Positively tested flocks have to be culled. In Switzerland, no cases of ILT occurred in

commercial poultry since 2006 (Personal communication, Hoop); in contrast several outbreaks are regularly detected in backyard flocks (www.infosm.bvet.admin.ch). The main host for infections with GaHV-1 represents barn fowl (Guy and Garcia 2008). Furthermore, pheasants, pheasant-chicken crosses and turkeys are also susceptible (Guy and Garcia 2008). Reports of virus isolation from tracheal tissue of peafowl and subclinical infection with seroconversion in ducks exist (Guy and Garcia 2008). Sparrows, crows, starlings, guinea fowl, quail and others are not susceptible (Guy and Garcia 2008). Chickens between three weeks and two years of age are most frequently affected with a peak between 10 weeks of age and the begin of lay (Guy and Garcia 2008, Rolle and Mayr 2007). ILTV is excreted through conjunctival secretions, tracheal mucus or faeces (Guy and Garcia 2008, Rolle and Mayr 2007). Infection occurs after mucosal contact with viral particles (Guy and Garcia 2008, Rolle and Mayr 2007). Spread through living or dead vectors is described (Guy and Garcia 2008, Rolle and Mayr 2007). It is known that infected embryos die before eclosion, therefore vertical transmission and infection does not lead to carrier birds (Rolle and Mayr 2007).

Like other Alphaherpesviruses, GaHV-1 is able to establish latency in the trigeminal ganglion (Guy and Garcia 2008, Rolle and Mayr 2007). Latently infected animals do not show any clinical signs but can shed virus after reactivation of the infection (Guy and Garcia 2008, Rolle and Mayr 2007).

The incubation period lasts 6 – 12 days p.i. (Guy and Garcia 2008, Rolle and Mayr 2007) and clinical signs vary from mild respiratory symptoms to acute death (Guy and Garcia 2008, Rutz and Hoop 2005, Rolle and Mayr 2007). Specific antibodies are detectable 5 – 7 days p.i., reach a maximum around 21 days and can persist for a year or even longer (Bagust et al. 2000, Guy and Garcia 2008). The humoral immune response does not seem to play a major role in the defence of infection with the GaHV-1 since there exists a poor correlation between serum antibody titer and immune status (Bagust et al. 2000, Guy and Garcia 2008). Therefore, the cell-mediated immunity is supposed to be more relevant, but so far not much is known about that topic (Bagust et al. 2000, Guy and Garcia 2008).

2.4.4 Diagnosis

The diagnosis of infection with GaHV-1 is based on clinical signs as well as several laboratory methods. Histopathological examination of tracheal tissue reveals a

desquamative, necrotizing tracheitis typical for infection with the GaHV-1 (Guy and Garcia 2008). In addition, viral inclusion bodies can be detected (Guy and Garcia 2008). Beside pathological examination, classical virus isolation from inoculated cell culture or embryonated eggs, detection of antibodies by various serological tests and molecular based assays including the polymerase chain reaction (PCR) represent additional diagnostic procedures (Guy and Garcia 2008).

2.5 Aim of the study

The aim of this study is to evaluate the potential of LMH cells to analyse chicken Toll-like receptor function with special regard to the infection with GaHV-1.

3. Materials and Methods

3.1 Cell culture

LMH chicken hepatocellular carcinoma epithelial cells (ATCC CRL-2117, Kawaguchi et al. 1987) were used in all experiments. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX-I (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 1x antibiotic-antimycotic solution (SKU: 15240-062, Life Technologies, Carlsbad, CA, USA). Cultures were maintained at 37°C in a humified, 5% CO_2 incubator.

3.2 Viral infections

The infectious laryngotracheitis virus (ILTV) strain A489 was kindly provided by the Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany (Dr. Fuchs, Dr. Mettenleiter) and used for viral infections. Infection assays were done on LMH cells grown as described before. The virus was used in a multiplicity of infection (MOI) = 0.1. MOI is a virological term and means the ratio of the number of infection agents to the number of infection targets, meaning one infectious virus particle to 10 LMH cells in our experiments. Infected cells were grown at 37°C in medium containing 0% FCS and 1x antibiotic-antimycotic solution. The inoculum was not removed and at the indicated times, cells were processed for RNA extraction, Western Blot and ELISA. Cells were harvested after 2, 4, 6, 8, 24, 36 and 52 hours. The experiments were repeated three times.

ILTV stock production was done by propagating ILTV on LMH cells grown as described before. Harvest contained three freezing and thawing cycles. The titer of the virus was determined by standard $TCID_{50}$ determination assay on LMH cells. Stocks were stored in 1ml aliquots at -80°C. For each experiment, a fresh aliquot was thawed. As negative control (mock), uninfected LMH cells were treated the same way as the cells for virus stock production.

3.3 Treatment of cells with TLR agonists

The TLR agonist kit ('TLR1-9 Agonist Kit Human', Catalog ♯ tlrl-kit 1 hw, InvivoGen, San Diego, CA, USA) was used to analyse the change of the mRNA concentration in LMH cells watching TLR 3, 7, 21 and IL-6 and 8. The kit contains ten agonists for

human TLR 1-9, for each TLR one and for TLR 3 two different agonists. All agonists were used in maximum working concentration following the manufacturer's instructions. Harvest followed after 24 hours. A separate assay was done using only Lipopolysaccharide (LPS, 'TLR1-9 Agonist Kit Human', InvivoGen, San Diego, CA, USA) in maximum working concentration to examine the IL-6 response at different times.

3.4 Development and application of real-time polymerase chain reaction (PCR) assays

3.4.1 RNA extraction and reverse transcription into cDNA

RNA was isolated by the RNA-Extraction Kit NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Concentration as well as purity of the extracted RNA was determined using the NanoDrop® 2000c Spectrophotometer (Witec AG, Littau, Switzerland). Extracted RNA was stored at - 80°C. For cDNA-assembly, the Reverse Transcription System (Promega, Madison, WI, USA) was used. 1ug of total RNA was reverse transcribed, using random primers, according to the manufacturer's protocol with the exception of the hybridization time of the primers that was prolonged to 50 minutes. The obtained first-strand cDNA was stored at -20°C until use.

3.4.2 Primer design

Primer and probe sequences (Table 1) were designed with Primer3 software (Rozen and Skaletsky 2000) for TLR 3, 7 and 21 and with Primer Express software, Version 3.0 (Applied Biosystems by Lifetechnologies, Carlsbad, CA, USA) for IL-6 and IL-8. Primers were designed based on the gene sequences encoding the accordant protein (Accession numbers: table 1). Primers for the amplification of the avian GAPDH were chosen based on accession number M_11213 (Schybli 2010). Primers and probes were synthesized by Microsynth (Balgach, Switzerland).

3.4.3 Conventional PCR

A conventional PCR was performed to test the primers. The reaction mix contained 12.5ul RedTaq®ReadyMix™ (Sigma-Aldrich, St. Louis, MO, USA), 1ul (400nM) forward primer, 1ul (400nM) reverse primer (primers: table 1), 5ul cDNA or DEPC (=

diethylpyrocarbonate) treated water and 5.5ul DEPC treated water for one sample. For β-actin (TaqMan® β-actin Control Reagents, Applied Biosystems by Life Technologies, Carlsbad, CA, USA), the internal control, 12.5ul RedTaq®ReadyMix™, 3.3ul (400nM) forward primer, 3.3ul (400nM) reverse primer, 5ul cDNA or negative control and 0.9ul DEPC treated water was used. Amplification was performed on a Thermal Cycler 2720 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The cycling parameters were as follows: 3 min at 93°C for initial denaturation followed by 35 cycles of 1 min at 93°C, 1 min at 55°C, 1 min 72°C with a final elongation step for 10 min at 72°C. PCR products were analysed by gel electrophoresis using a 2% agarose gel. Gel documentation was performed by UV fluorescence measuring with an AlphaInnotech system and AlphaImager software (AlphaInnotech by ProteinSimple, Santa Clara, CA, USA).

3.4.4 Plasmid construction for IL-6, IL-8, TLR 3, 7 and 21 standard curve analysis

The PCR products obtained with conventional PCR were cloned into a pCR™2.1-TOPO® vector using the TOPO® TA Cloning® Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Incubation at room temperature was prolonged to 30 minutes. For transformation of the competent cells, the regular chemical transformation protocol provided by the manufacturer was used. Incubation on ice was prolonged to 30 minutes and ampicillin containing LB agar plates (Ampicillin 100ug/ml) were used for bacterial cultivation. After over night incubation, 10 of the grown colonies were picked and used for plasmid minipreparation using the standard alkaline lysis protocol (Green and Sambrook 2012). Obtained plasmid DNA was digested with EcoR I enzyme (New England Biolabs, Ipswich, MA, USA). Two clones showing the expected restriction enzyme pattern on a 2% agarose gel were chosen and sent for sequencing to Microsynth (Balgach, Switzerland). Concentration and purity of the isolated plasmid DNA was determined using the NanoDrop (Witec AG, Littau, Switzerland).

3.4.5 Analytical sensitivity and specificity of the newly designed real-time PCR assays

Real-time PCR

The reaction mix was composed as follows: 12.5ul TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 1ul (400nM) forward primer, 1ul

(400nM) reverse primer, 0.5ul probe (200nM) (table 1) and 5ul DEPC treated water per sample. 5ul of cDNA or DEPC water as a negative control was added to 20ul of the reaction mix. Cycling program was performed by the real-time PCR System 7500 Fast (Applied Biosystems by Life Technologies, Carlsbad, CA, USA): 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All samples were tested in duplicates. Results were analysed with the program 7500 Fast System SDS Software version 1.4.0 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). The threshold values, using the detectors FAM and TAMRA, were set at 0.05 for IL-6 and at 0.1 for IL-8, TLR 3, 7, 21 and GAPDH, the housekeeping gene.

Standard curve analysis

Tenfold serial dilutions of the constructed plasmids ranging from 10^{-3} to 10^{-11} were made in DEPC water and used for creation of the standard curve. Undiluted plasmid concentrations are shown in table 2. Real-time PCR amplification was carried out as described above and the experiment was repeated three times.

3.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for the detection of chicken IL-6 and IL-8 were used (Cusabio, Hölzel Diagnostic, Köln, Germany). Supernatants from ILTV-infected, mock or TLR agonist treated cells were used for analysis. Assays were performed according to the kit instructions. The IL-6 ELISA follows the direct ELISA principle, IL-8 is measured in a competitive procedure. Briefly, samples, standard and blank are added to the appropriate microtiter plate wells. For IL-6, the plate has been pre-coated with an antibody specific to IL-6. A biotin-conjugated antibody and, subsequently separated through washing steps, avidin conjugated to Horseradish Peroxidase (HRP) is added to the wells. Together with TMB (3.3'.5.5'tetramethyl-benzidine) substrate, those wells containing IL-6, exhibit a change in colour which can be measured. Concerning the IL-8 ELISA, plates have been pre-coated with IL-8 antigen. Samples together with a HRP conjugated antibody specific for IL-8 are added to the wells. After washing, the TMB substrate is added and dependent on the amount of IL-8 concentration in the samples, a change in colour is detectable. The intensity of colour change is indirectly proportional to the amount of IL-8 present in the sample. Optical

density (OD) was determined by using the microplate reader Multiskan Ex Primary EIA V.2.3. (Thermo Fisher Scientific, Waltham, MA, USA) and results were converted with Ascent Software version 2.6. Standard curve fit was done in 'log-logit' mode.

3.6 Western Blot analysis

LMH cells were plated in 25cm^2 cell culture flasks in a concentration of 4 x 10^6 cells per flask. The first 24 hours, cells were grown in medium containing 10% FCS, the following 24 hours, cells were prepared to infection in 0% FCS medium. Infection was done as described before. At different time points, cells were washed with ice-cold PBS and lysed in RIPA buffer (Radio Immuno Precipitation Assay buffer, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). 50ul of total protein was loaded on 10%-SDS polyacrylamidgels and separation was performed following the standard SDS PAGE protocol (Laemmli 1970). Proteins were transferred to a nitrocellulose transfer membrane Protran® (Whatman®, Kent, UK) and blocked in 5% non-fat dry milk in Tris-Buffered Saline (TBS) for 1 hour at room temperature. Membranes were first incubated over night at 4°C with a rabbit-anti-chicken IL-6 antibody (AbD Serotec, Kidlington, UK) diluted 1:1000 in 1% non-fat dry milk in TTBS buffer (TBS buffer containing 0.05% Tween20). After three wash steps using TTBS, a goat-anti-rabbit IgG antibody (AbD Serotec, Kidlington, UK) was added to the membranes. The antibody was used in a 1:1000 dilution in 1% non-fat dry milk in TTBS buffer. The membranes were incubated 2.5h at room temperature. For positive control 1.25ug of recombinant chicken IL-6 protein (AbD serotec, Kidlington, UK) was used. Subsequently, blots were washed again as described above. A homemade enhanced chemiluminescence (ECL) protocol was performed for the detection of the bound antibodies. Briefly, 1 ml solution A (500 ml 0.1M TRIS-HCl (pH 6.8), 125mg Luminol sodium salt A4685-1G, Sigma-Aldrich, St. Louis, MO, USA) was mixed with 0.3ul 35% H_2O_2 and 100ul solution B (11 mg para-hydroxycoumarin acide (Sigma-Aldrich, St. Louis, MO, USA) in 10 ml dimethyl sulfoxide). As internal control, the same blots were reused with the mouse anti actin MAb clone AC-74 (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10 000 in the same procedure as with the IL-6 antibody. To visualize the chemiluminescence, Fuji Medical X-ray Film 100 NIF (Fujifilmcorporation, Tokyo, Japan) was exposed to the membranes. Film development was performed by the Kodak X-Omat 2000 Processor (Eastman Kodak Company, Rochester, NY, USA).

4. Results

4.1 Analysis of TLR 3, 7 and 21 mRNA expression in LMH cells by newly established quantitative real-time PCR assays

Conventional PCR revealed bands with the correct size for all three TLRs (Figure 1, lanes 1-6, amplicon size: table 1). In quantitative real-time PCR assays the linear regression analysis demonstrated a correlation between the cycle threshold (ct) value and the amount of the plasmids over a 7-10-log range (Figure 2a-c). All internal controls delivered correct results.

4.2 Analysis of IL-6 and IL-8 mRNA expression in LMH cells by newly established quantitative real-time PCR assays

Testing the primers as described above showed, that mRNA detection of IL-8 was successful in native LMH cells (Figure 1 lane 9, Figure 2e). In contrast, IL-6 mRNA detection failed in native LMH cells (Figure 1 lane 7) but was successful in ILTV-infected cells (Figure 2d, Figure 4a).

4.3 Analysis of Toll-like receptor mRNA expression in ILTV-infected and mock treated LMH cells

In conventional and real-time PCR, TLR 3 mRNA expression fluctuated around the ground level in mock treated and ILTV-infected cells. After 24 hours, a clear reduction of mRNA concentration by 77% in ILTV-infected cells was observed (Figure 3a, 3d). The mRNA expression of TLR 7 showed similar results, with the exception of mock treated cells taken at 6 hours p.i. and a slight increase in infected cells after 36 hours (Figure 3b, e). Concerning TLR 21 mRNA expression levels, no obvious change was seen in ILTV-infected cells (Figure 3c, f).

4.4 Analysis of Cytokine mRNA expression in ILTV-infected and mock treated LMH cells

4.4.1 Conventional and real-time PCR assays used for the detection of IL-6 and IL-8 mRNA

No IL-6 mRNA production was observed in all samples except in the ILTV-infected LMH cells later than 24 hours (Figures 4a and 4c). On the other hand, IL-8 mRNA

expression was detectable in all samples, in ILTV-infected as well as mock treated cells (Figures 4b and 4d). A clear increase of IL-8 mRNA concentration after 24 hours was visible.

4.4.2 Western blot analysis for the detection of IL-6

No IL-6 protein was detectable in any experiment.

4.4.3 Enzyme-linked-immunosorbent-assays for the detection of IL-6 and IL-8 protein

No detectable levels of IL-6 protein were present. In contrast, the IL-8 protein could be detected at every time point sampled (Figure 5 a-b). An increase of IL-8 protein was observed in mock treated cells after 24 hours. In contrast, a decrease of IL-8 protein appeared in ILTV-infected cells. The detection range of the ELISA kit reached from 6.25 – 400pg/ml.

4.5 Analysis of Toll-like receptor mRNA expression in LMH cells treated with Toll-like receptor agonists

All cells stimulated with the two TLR 3 agonists Poly I:C HMW and LMW detached from the culture flask. The medium from cells treated with TLR 7 agonist Imiquimod became yellow in contrast to the other cell cultures, nevertheless the cells looked normal. The cause for this change in colour is unknown. The results of the real-time PCR assays were inconsistent and no conclusion could be drawn. No IL-6 protein was detected using ELISA and Western Blot. The concentrations of IL-8 protein after treatment with different TLR agonists were measured with ELISA (Figure 6).

5. Discussion

The main goal of this work was the evaluation of LMH cell culture as an *in-vitro* system for the analysis of the chicken Toll-like receptor system. The first step to address this topic included the establishment of newly developed PCR assays. Both newly developed PCR systems, conventional and real-time, were successful for TLR 3, 7, 21 and IL-8 in native cells. To develop the PCR detecting IL-6, ILTV-infected LMH cells had to be used, because uninfected cells expressed no IL-6. This data corresponded and supported the findings of Prinz (2010) and Terasaka and coworkers (2010). Both found that feline PBMCs (peripheral blood mononuclear cells), Crandell Reese Feline Kidney cells (Prinz 2010) and Human Corneal Epithelial cells (Terasaka et al. 2010) expressed IL-6 only after stimulation with a Herpesvirus or Toll-like receptor agonist. The newly developed real-time PCR assays detected 22 copies in all experiments. Amplification of TLR 3 and 7 was even successful with just 3 plasmid copies. Minimal detection range for IL-6 and TLR 21 were 22 and for IL-8 16 copies. Herewith the establishment of the new conventional and real-time PCR assays was completed and a novel tool for the analysis of LMH cells was created.

In a next step, the existence of TLR 3, 7 and 21 mRNA as well as the presence of IL-6 and IL-8 mRNA was analysed in native LMH cells over 24 hours, because the TLR repertoire differs from cell type to cell type according to its function (Boehme and Compton 2004). Detection of mRNA for TLR 3, 7 and 21 was successful in all samples. The levels of the mRNA concentrations stayed around the basic level determined at 2 hours (data not shown). Analysing IL-8 PCR results showed, that IL-8 mRNA is detectable in all samples and increased constantly to a 5 to 6 fold over 24 hours (data not shown). The change in IL-8 mRNA concentration without an external stimulus could be explained due to the divers functions of IL-8. As described by Sick and coworkers (2000) and Martins-Green and Feugate (1998), IL-8 acts as a chemotactic and angiogenic factor and is also involved in growth regulation and wound healing. As expected no IL-6 mRNA was detected in native LMH cells.

Having shown the presence of TLR 3-, 7- and 21- as well as IL-8 mRNA in LMH cells, we further wanted to study the TLR system in LMH cells by various tools. In a first set of experiments, cells were infected with ILTV. The interpretation of the infection experiments was difficult because there were outliers, but however, we gained valid data. Regarding the TLR 3 mRNA concentration, it decreased after 24 hours and stayed on low levels as shown by real-time PCR. Interestingly, the mRNA

level of TLR 7 decreased at 24 hours similar to TLR 3, but then increased again and reached 145% of the level determined at 24 hours in mock treated cells. The study by Li and coworkers (2005) assumed a sequential activation of TLRs after infection to modulate the immune response. From the results of our study and the study by Khvalevsky and coworkers (2007) we hypothesize that TLR 3 activation led to partial apoptosis in LMH cells resulting in a lower mRNA concentration. It is known that Herpesviruses are capable to manipulate the host's immune system to its own benefit. So Epstein-Barr virus is able to influence TLR 7 and Kaposi's sarcoma associated Herpesvirus can modify TLR 3 (Martinez-Martin and Viejo-Borbolla 2010). Infection of LMH cells with ILTV had no visible influence on TLR 21 mRNA concentration. MRNA concentration of the cytokines IL-6 and IL-8 increased significantly at 24 hours p.i. Until 52 hours p.i. IL-6 mRNA concentration continued to increase whereas mRNA concentration of IL-8 decreased. LMH cells showed a clear innate immunological reaction to infection with ILTV. To study if the cytokine mRNA was successfully translated into proteins, Western blot for IL-6 and ELISA for IL-6 and IL-8 were performed. An IL-8 Western blot analysis was planned, but no chicken IL-8 antibody was available. IL-8 protein could be detected in all samples using ELISA. Including the time period from mRNA expression to protein translation, ELISA results confirm the PCR assay results for IL-8. Protein detection of IL-6 succeeded neither in Western blot nor in ELISA. Regarding the cycle threshold, we presume a too low concentration of IL-6 protein in cell lysates and cell culture supernatant.

In order to test the functionality of LMH cells by various tools, the cells were treated with known TLR agonists. The effect of human TLR agonists on chicken TLRs was tested because we assumed a similarity of the TLR pathways in chickens and mammalians. Although the PCR assays revealed no conclusive results, an influence of TLR agonists on LMH cells is present regarding the microscopical effects (see 4.5). Our results using TLR 3 agonists poly I:C HMW and LMW are in accordance with the findings of Khvalevsky and coworkers (2007), who demonstrated apoptosis in a human hepatoma cell line upon treatment with TLR agonist poly I:C. It is reported that the activation of the TLR 3 pathway can have variable effects depending on which virus triggers TLR 3 and in which cell type the receptor is expressed (Vercammen et al. 2008). For example the infection of TLR 3-deficient and wild-type mice with Influenza A virus revealed a lower survival rate in wild-type mice because of the detrimental inflammation reaction mediated through TLR 3

(Vercammen et al. 2008). Khvalevsky and coworkers (2007) assumed a related explanation for the apoptosis in HepG2 cells, namely that TLRs are capable to direct the infected cells either to the secretion of proinflammatory cytokines or to its elimination through apoptosis, depending on the use for the organism. For further study it would be valuable to separate the apoptotic cells from healthy cells and debris using propidiumiodide staining and fluorescence activated cell sorting (FACS) as described in Riccardi and Nicoletti (2006).

Interestingly no IL-6 mRNA or protein was detectable in any sample stimulated with TLR agonists, not even in LPS-stimulated LMH cells. LPS is a reliable and potent activator for TLR 4 and in consequence of IL-6 expression (Hoshino et al. 1999, Lu et al. 2008). We assumed a potential deficient NFκB protein in LMH cells and therefore the inability to trigger IL-6 over the NFκB dependent TLR 4 pathway. In opposite, ILTV is able to trigger not only one receptor at once but interacts with different mechanisms of the innate immunity. For the virus it seems possible to induce IL-6 over NFκB independent pathways as for example the TLR 3 pathway (Takeda and Akira 2004). Variable influences modulate the mRNA concentration of IL-6 (Chen and Manning 1995, Dokter et al. 1996, Hoshino et al. 1999, Nishimichi et al. 2005, van Duin et al. 2007). According to the ability of Herpesviruses to assimilate host's genes through recombination and adaptation (Moore et al. 1996, Parcells et al. 2001, West and Damania 2010), one could postulate a viral IL-6 homologue. Using the BLAST function on NCBI's webpage we compared IL-6 cDNA sequence (HM_179640, 729 bp) and cDNA sequence of the IL-6 precursor (AJ_250838) with the first complete genome sequence of GaHV-1 serva vaccine strain (Lee et al. 2011). No homologues were found apart from short analogies (around 30 bp).

Overall conclusion

The evaluation of LMH cells to study chicken Toll-like receptor functions using ILTV and Toll-like receptor agonists showed, that LMH cells are functional but not perfectly suitable. The growth pattern of the cells was irregular, cultivation sometimes failed completely and the cells showed signs of obsolescence after on average 30 passages. In combination with ILT virus, it was very difficult to reach good $TCID_{50}$ values in virus stock production. We also assume a malfunction in cell internal pathways concerning NFκB and maybe other proteins, which needs to be

investigated. Nevertheless, this study delivered interesting results concerning the interaction between ILTV and LMH cells and showed deficits in the use of LMH cell line. In conclusion, one can say, that the LMH cell line is an alternative to the primary, embryonic cell lines within its limitations. They provide potential for further research.

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Description to Figures and Tables

Figure 1

Testing the primers for TLR 3, 7, 21, IL-6 and IL-8 on native LMH cells,
amplicon sizes: see table 1
Ma: 100bp DNA ladder (Invitrogen by Lifetechnologies, Carlsbad, CA, USA)

Figure 2 (a-e)

2a-c, 2e: Standard Curve Analysis for TLR 3, 7 and 21 and IL-8 on native cells
2d: Standard Curve Analysis for IL-6 on ILTV-infected cells

Figures 3 (a-g)

Figures showing real-time PCR results: ground level determined at 2 hours
3a: TLR 3 detection in mock treated (M) and ILTV (ILT) infected LMH cells
3b: TLR 7 detection in mock treated (M) and ILTV (ILT) infected LMH cells
3c: TLR 21 detection in mock treated (M) and ILTV (ILT) infected LMH cells

Figures showing real-time PCR results: ground level determined at 24 hours
3d: TLR 3 detection in mock treated (Mock) and ILTV (ILT) infected LMH cells
3e: TLR 7 detection in mock treated (Mock) and ILTV (ILT) infected LMH cells
3f: TLR 21 detection in mock treated (Mock) and ILTV (ILT) infected LMH cells

Figures 4 (a-e)

Figures showing conventional PCR results
4a: IL-6 detection in ILTV-infected LMH cells (duplicates)
4b: IL-8 detection in ILTV-infected LMH cells (duplicates)

Figures showing real-time PCR results: ground level determined at 24 hours
4c: IL-6 detection in mock treated (Mock) and ILTV (ILT) infected LMH cells
4d: IL-8 detection in mock treated (Mock) and ILTV (ILT) infected LMH cells

Figures 5 (a-b)

5a: ELISA detecting IL-8 in ILTV-infected cells
5b: ELISA detecting IL-8 in mock treated cells

Figure 6

ELISA detecting IL-8 in TLR agonist treated cells after 24 hours, TLR agonists 1 – 9 used

Figures and tables

Table 1: Primers and probes

Oligonucleotid	Accession no	Sequence	Amplicon size
For_IL-6	HM_179640	5’ GCT TCG ACG AGG AGA AAT GC 3’	169 bp
Rev_IL-6		5’ CCG GCA GAT GGT GAT AAA TCC 3’	
Probe_IL-6		5’ TGA CGA AGC TCT CC 3’	
For_IL-8	NM_205498	5’ GCA AGG TAG GAC GCT GGT AAA G 3’	159 bp
Rev_IL-8		5’ GTT GAA ATC ATA GCT ACT CTA AAG GAT GG 3’	
Probe_IL8		5’ ATG AGC TGC GGT GCC 3’	
For_TLR 3	XM_001231986	5’ ACG TGT TTG GTC CAG CTT TC 3’	110 bp
Rev_TLR 3		5’ AGC CAA CTA GCA AAC CAA GC 3’	
Probe_TLR 3		5’ CCA TTT GAT TGC ACC TGT GAA AGC A 3’	
For_TLR 7	AJ_632302	5’ GAA TTC AAG AGG TTC AGG AAC ATG A 3’	118 bp
Rev_TLR 7		5’ TTA GGG CAG GGA GTA CAA GGA TAT 3’	
Probe_TLR 7		5’ CAA TTG CCC ACG TTG CTA TAA CGC CC 3’	
For_TLR 21	NM_001030558	5’ AGG TGT TGT GGC TCA ATA TTA ACA G 3’	143 bp
Rev_TLR 21		5’ CAG TCT GTG GAG GTC AAT GAA G3’	
Probe_TLR21		5’ ACA ACC TGC TGA CCG ACC TCT ATC ACA ACT 3’	

Table 2: Undiluted plasmid concentrations

Plasmid	Concentration (copies per ul)
pIL-6	2.12×10^{11}
pIL-8	1.54×10^{11}
pTLR 3	2.93×10^{11}
pTLR 7	2.87×10^{11}
pTLR 21	2.19×10^{10}

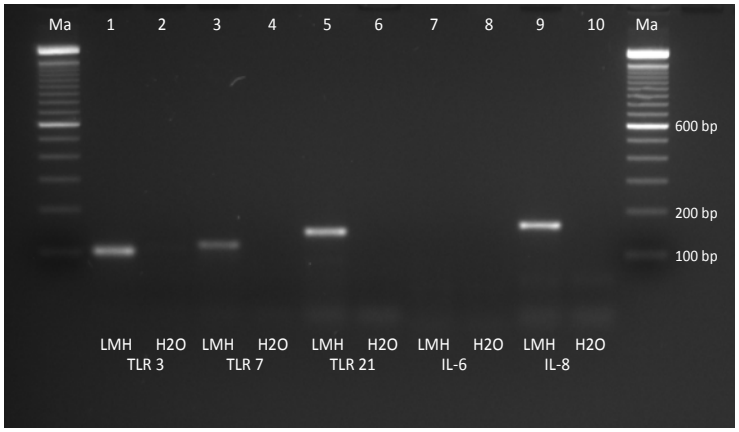
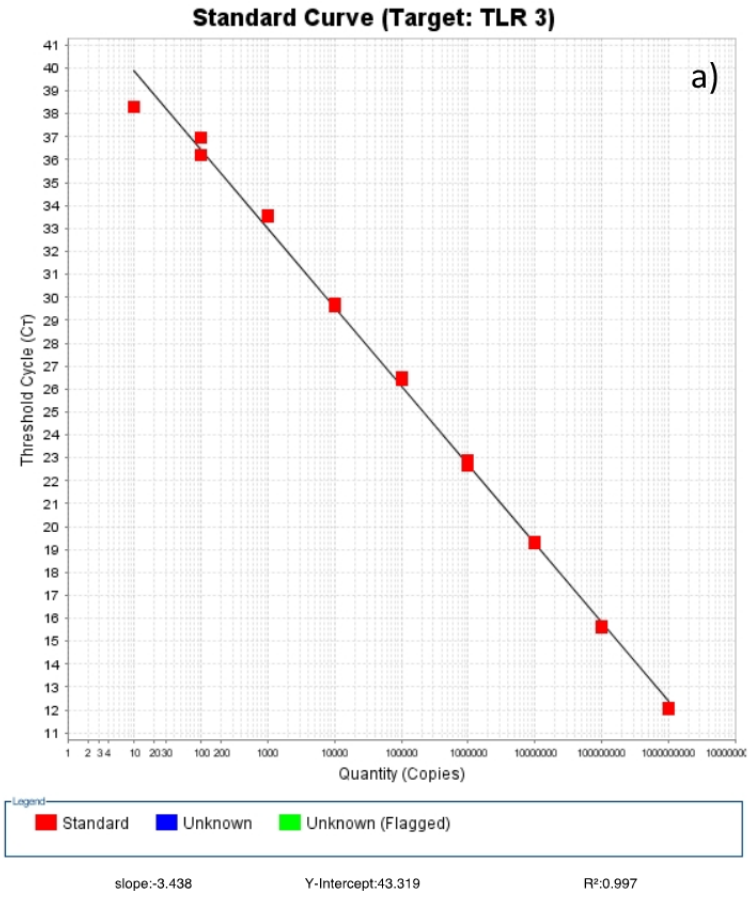
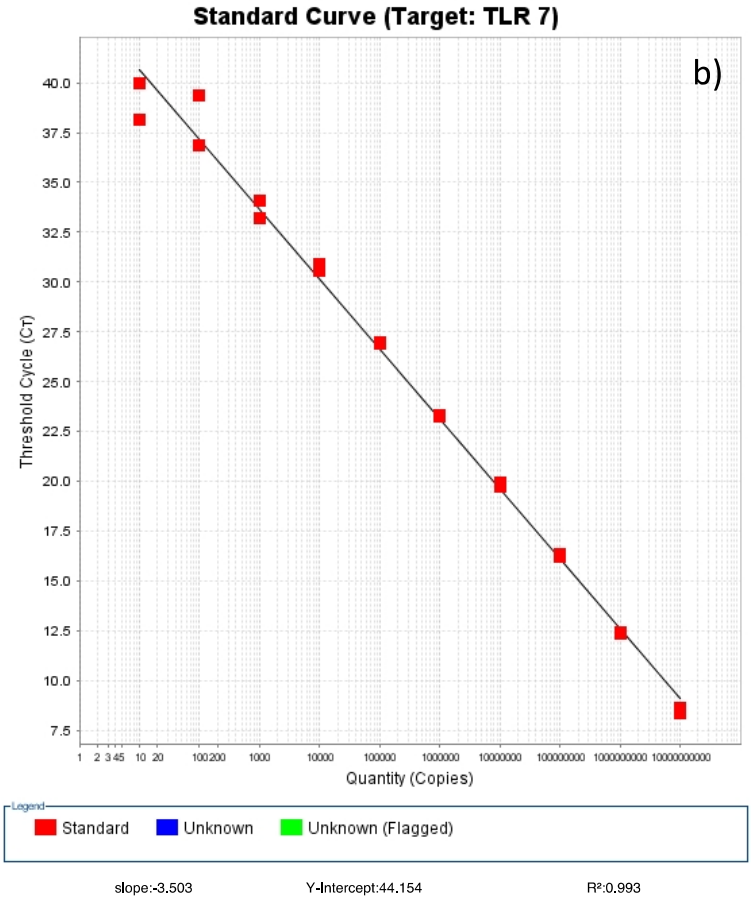


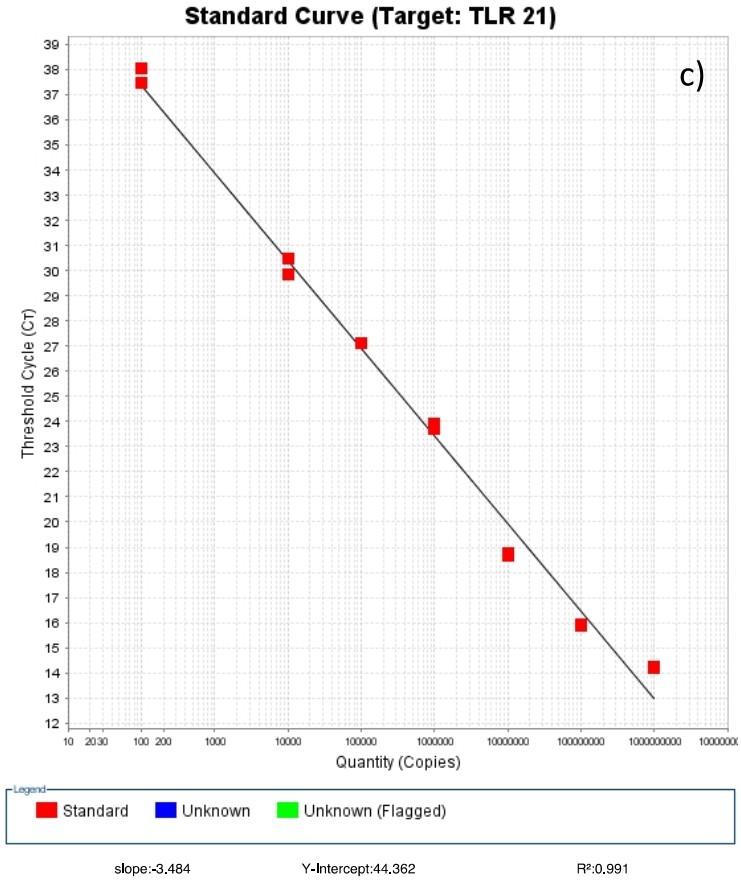
Figure 1

Figures 2 (a-e)

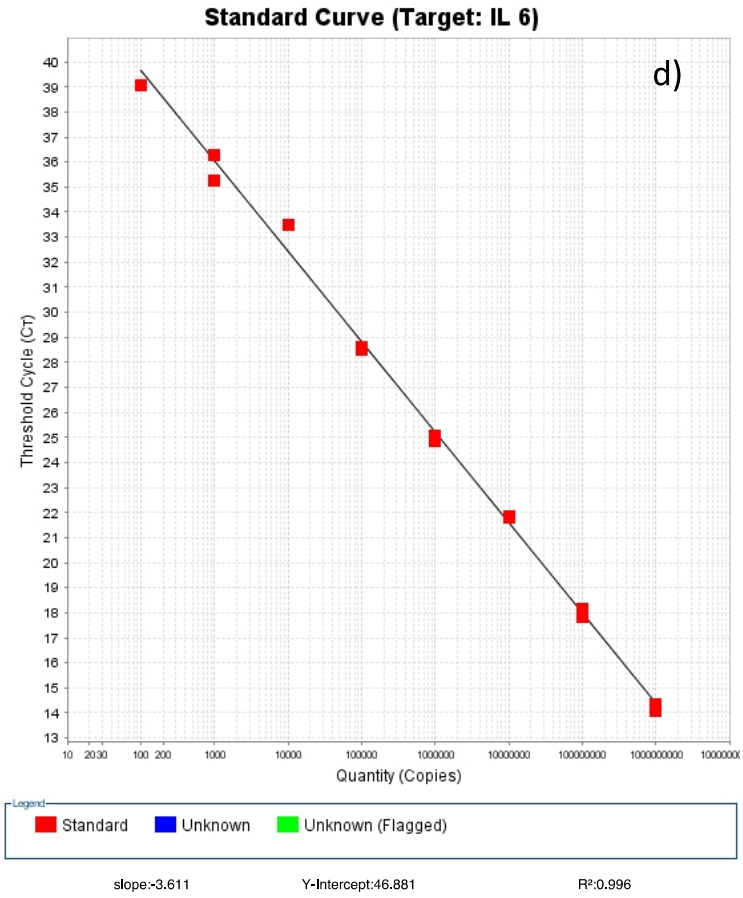




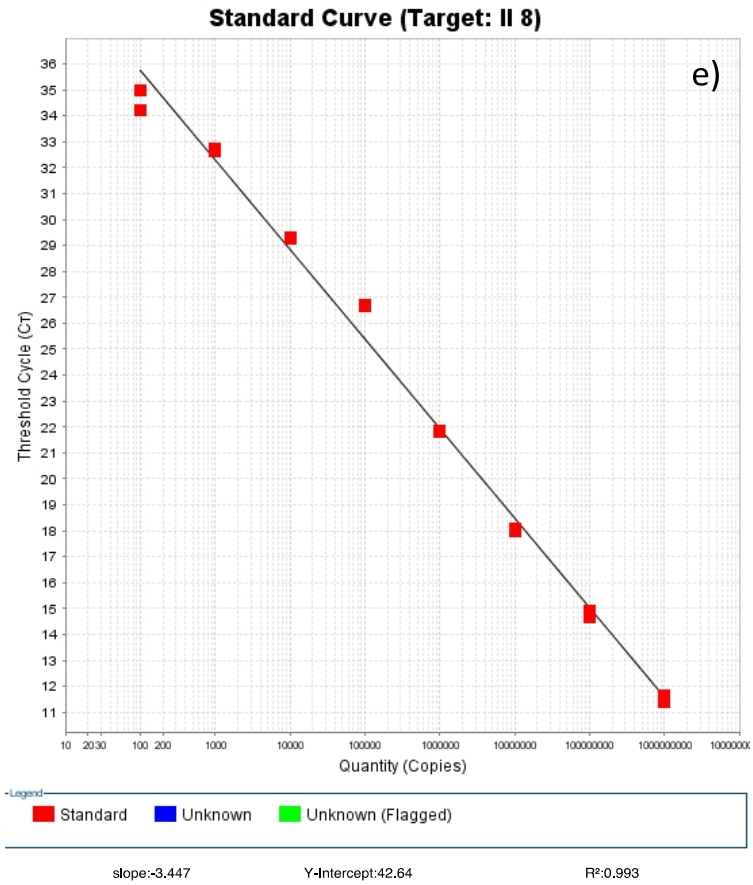
slope:-3.503 Y-Intercept:44.154 R²:0.993



slope:-3.484 Y-Intercept:44.362 R²:0.991



slope:-3.611 Y-Intercept:46.881 R²:0.996



slope:-3.447 Y-Intercept:42.64 R²:0.993

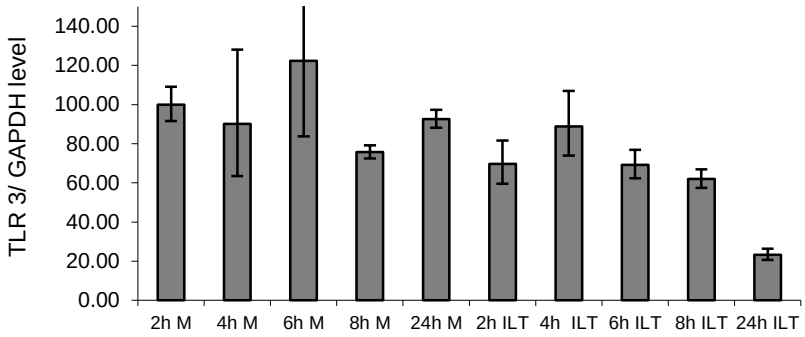


Figure 3a

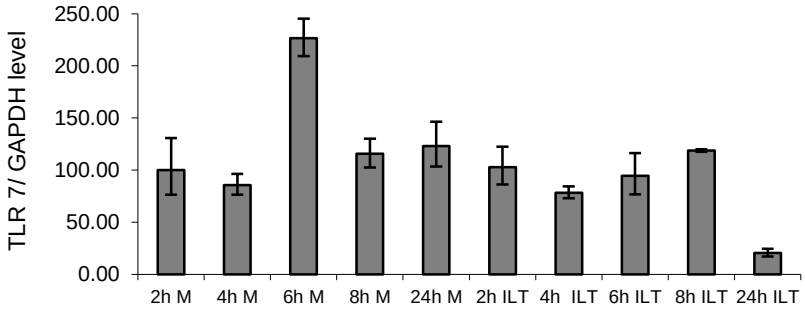


Figure 3b

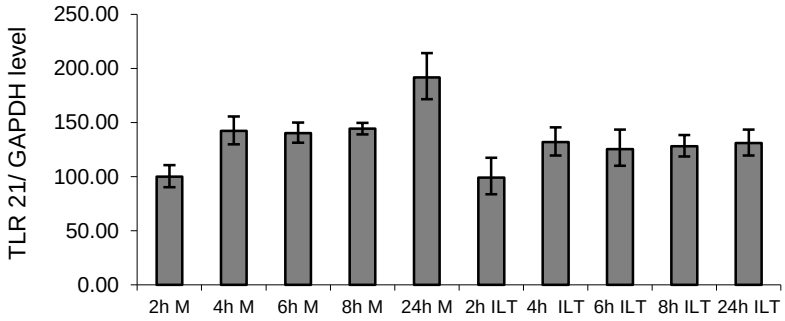


Figure 3c

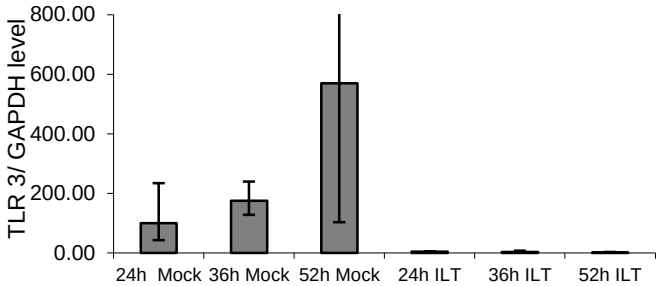


Figure 3d

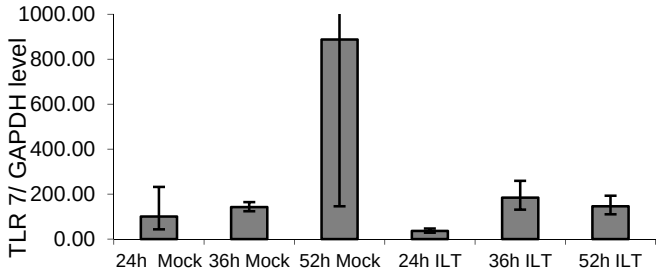


Figure 3e

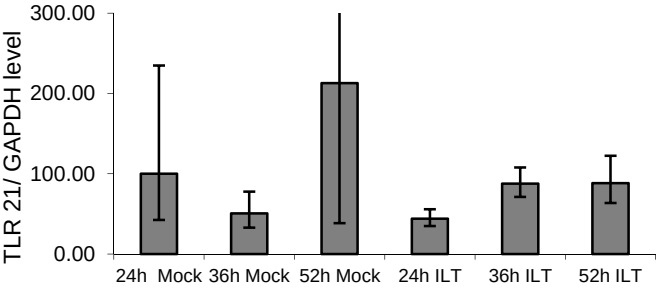


Figure 3f

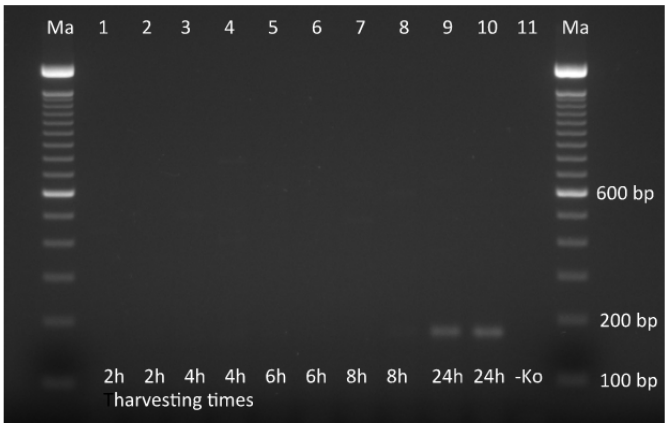


Figure 4a

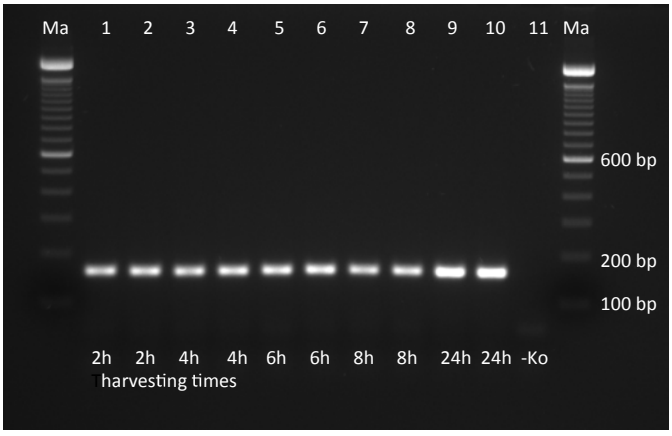


Figure 4b

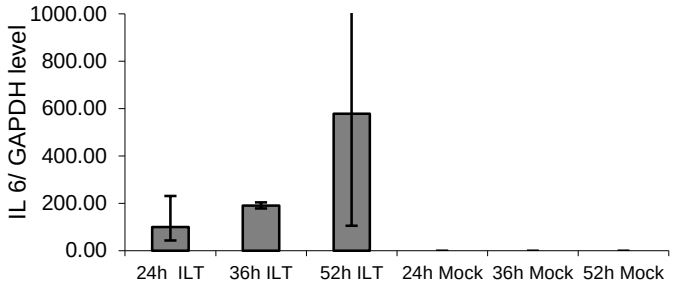


Figure 4c

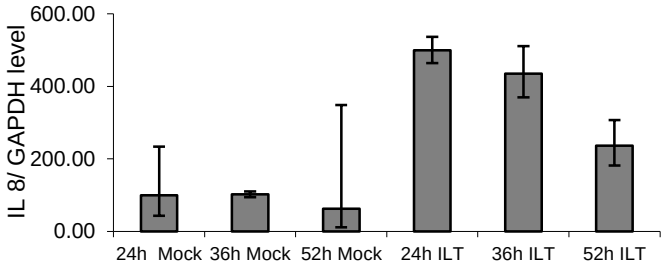


Figure 4d

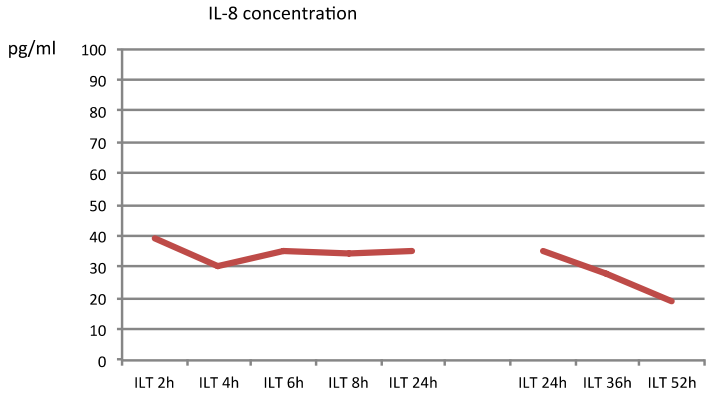


Figure 5a

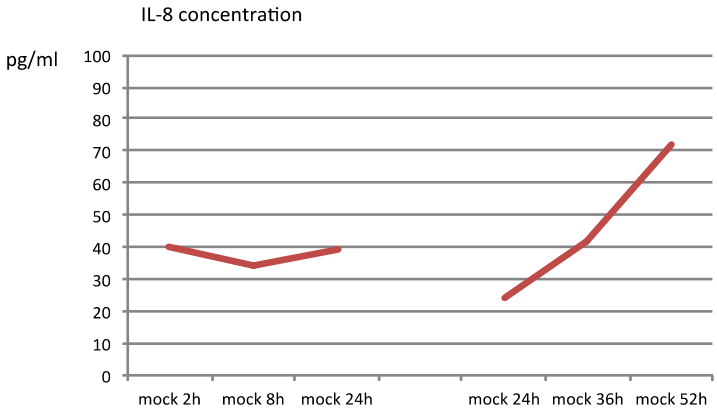


Figure 5b

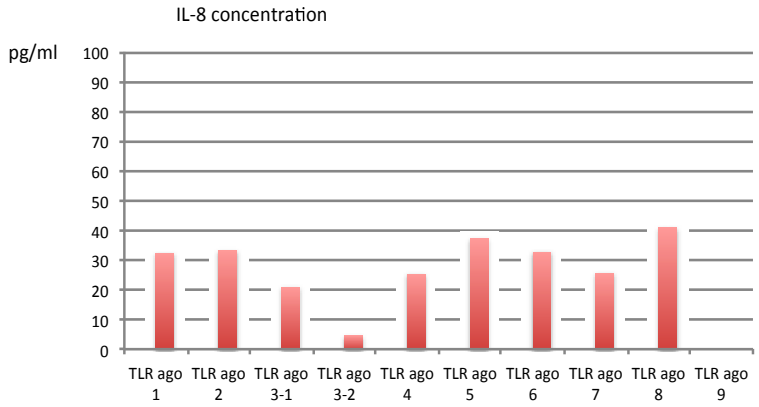

IL-8 concentration
pg/ml
100
90
80
70
60
50
40
30
20
10
0
TLR ago 1
TLR ago 2
TLR ago 3-1
TLR ago 3-2
TLR ago 4
TLR ago 5
TLR ago 6
TLR ago 7
TLR ago 8
TLR ago 9

Figure 6

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